Calcium channel antagonist binding sites labeled by $^3$H-nimodipine in human brain

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In vitro binding of $^3$H-nimodipine to human brain membranes is demonstrated in this study. This binding was specific and saturable, and had an apparent affinity constant ($K_D$) of 0.27 nM. The maximal number of binding sites for $^3$H-nimodipine was 5.8 pmol/gm wet weight of human frontal cortex. The binding was shown to be dependent on calcium, with half-maximal stimulation obtained at $3 \times 10^{-5}$ M CaCl$_2$. Other 1,4-dihydropyridine calcium antagonists were shown to be competitive antagonists of $^3$H-nimodipine binding. In contrast, the calcium antagonists, verapamil and diltiazem, had complex interactions with $^3$H-nimodipine binding. These results represent the first identification of $^3$H-calcium antagonist binding sites in human brain, and they confirm that various calcium antagonist drugs may differ with respect to both their potency and their molecular site of action.

KEY WORDS  
- nimodipine  
- calcium antagonist  
- receptors  
- vasospasm  
- cerebral arterial spasm

Calcium channel antagonists are a recently developed group of vasodilators that inhibit the influx of calcium through receptor- or voltage-operated ion channels in cell membranes. Initially, the clinical use of these drugs was limited to the treatment of cardiovascular disorders such as vasospastic angina, arrhythmias, and hypertension. Recently, however, a role for calcium antagonists has also been proposed in the treatment of the cerebral arterial spasm that often follows a subarachnoid hemorrhage (SAH). In recently completed clinical trials, the calcium antagonist, nimodipine, was found effective in preventing symptomatic vasospasm following SAH.

Radioligand studies have been used to characterize the binding sites labeled by $^3$H-calcium antagonists in a number of tissues and species. Indeed, three distinct classes of calcium antagonists have been described in non-human tissues on the basis of radioligand studies. The presence of calcium antagonist receptor sites in human tissues, however, has not yet been documented. In the present report, we describe the specific and saturable binding of $^3$H-nimodipine in human brain membranes. The pharmacological characteristics of this site suggest an association with calcium channels in human brain tissue.

Materials and Methods

Human frontal cortex was obtained at autopsy within 12 hours of death. Cortical samples were dissected free of meninges, frozen over dry ice, and stored at $-70^\circ$C until used in assays within the next 3 weeks. On the day of experimental use, a 1- to 2-gm sample of tissue was placed at room temperature for 1 hour and suspended in 50 vol of 50 mM Tris-HCl buffer (pH 7.7 at 25$^\circ$C) using a Tekmar polytron for 10 seconds. The tissue suspension was then centrifuged at 50,000 G for 10 minutes, resuspended in 50 vol of Tris-HCl buffer, and again centrifuged at 50,000 G for 10 minutes. The pellet was again suspended in 80 vol of the standard assay buffer which included 50 mM Tris-HCl, 4 mM CaCl$_2$, and 0.1% ascorbic acid. Final tissue concentration was 10 mg wet weight/ml. Tissue used in the calcium regulation studies was initially suspended in 50 mM Tris-HCl and 5 mM ethylenediaminetetraacetic acid (EDTA) at 37$^\circ$C for 5 minutes prior to the standard tissue preparation described above.

Incubation tubes received 100 $\mu$l of $^3$H-nimodipine, 100 $\mu$l of various drugs, and 0.8 ml of tissue suspension during standard assays. All assays were performed in triplicate. The concentration of $^3$H-nimodipine in drug displacement studies was 0.1 nM. The tubes were incubated at 25$^\circ$C for 1 hour and then the contents rapidly filtered under vacuum through Whatman GF/B filters with three 5-ml washes of ice-cold 50 mM Tris-HCl buffer. The filters were counted by liquid scintillation spectrometry in 10 ml of Ultraflor at efficiencies of 40% to 44%.

Specific binding of $^3$H-nimodipine was defined as the
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Fig. 1. 3H-nimodipine binding to human frontal cortex membranes as a function of increasing concentrations of 3H-nimodipine. A: Frontal cerebral cortex membranes were incubated under standard assay conditions with increasing concentrations of 3H-nimodipine. Nonspecific binding (circles) was defined as the amount of 3H-nimodipine found in the presence of 100 nM nifedipine. Specific binding (squares) represents the difference between total (asterisks) and nonspecific binding. The experiment was performed in triplicate and repeated four times. B: Scatchard analysis of the data in A. KD = apparent affinity constant; BMAX = maximal number of binding site for 3H-nimodipine.

Results

3H-Nimodipine Binding to Human Brain

The binding of 3H-nimodipine to human brain membranes is saturable (Fig. 1A). Specific binding accounts for approximately 70% to 80% of total binding at concentrations below 0.1 nM 3H-nimodipine, the amount of 3H-ligand used in routine drug displacement studies. At concentrations above 0.1 nM, specific binding represents a decreasing percentage of total binding, and is only 45% of total binding at 1.0 nM 3H-nimodipine. The apparent monophasic saturation curve for 3H-nimodipine is confirmed by Scatchard analysis shown in Fig. 1B. Linear regression analysis reveals a correlation coefficient of 0.98 (p < 0.01) with the maximal number of binding sites for 3H-nimodipine equal to 5.8 pmoles/gm wet weight human frontal cortex. The apparent affinity constant (KD) of 0.27 nM is similar to the reported affinity of nimodipine for binding sites in non-human brain membranes.\(^{9,13,16}\)

Regulation of 3H-Nimodipine Binding by Calcium Chloride

Human frontal cortex binding of 3H-nimodipine is dependent on calcium (Fig. 2), a property shared with 3H-calcium antagonist binding sites in non-human tissues.\(^{13,14}\) After pretreatment with EDTA, specific binding of 3H-nimodipine is reduced to 24% of control values taken under standard assay conditions (that is, in the presence of 4 mM CaCl₂). The addition of 10⁻⁶M CaCl₂ causes a slight increase in specific binding, with maximal stimulation of 3H-nimodipine binding occurring at 1 mM CaCl₂. Specific binding is essentially stable at between 1 and 100 mM of calcium. Half-maximal stimulation is obtained at approximately 3 x 10⁻⁵M CaCl₂. Pretreatment with EDTA had no effect on nonspecific binding (data not shown).

Drug Effects of 3H-Nimodipine Binding to Human Brain

Nimodipine, nitrendipine, and nifedipine are all potent inhibitors of 3H-nimodipine binding (Fig. 3). Each of these 1,4-dihydropyridine calcium antagonists displays monophasic inhibition of 3H-nimodipine binding with Hill slopes of approximately 1.0. Nimodipine and nitrendipine are equipotent, with nifedipine being slightly less active in displacing 3H-nimodipine. This

* Drugs were obtained from the following sources: nimodipine, nifedipine, and nitrendipine from Miles Pharmaceuticals, 400 Morgan Lane, West Haven, Connecticut; sodium nitroprusside from Roche Laboratories, Nutley, New Jersey; propranolol from Ayerst Laboratories, 685 Third Avenue, New York, New York. Diltiazem and verapamil were the generous gifts of Dr. Solomon H. Snyder.
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**Fig. 2.** Effect of calcium chloride on $^3$H-nimodipine binding in human frontal cortex. Frontal cerebral cortex membranes were pretreated with 5 nM EDTA prior to incubation with increasing concentrations of CaCl$_2$. Data given are the means of triplicate assays performed in a single experiment. Each experiment was replicated four times.

Type of competitive antagonism among the 1,4-dihydropyridines has been noted in a variety of other tissues.$^{5,13}$ In marked contrast, the interactions of verapamil and diltiazem with $^3$H-nimodipine binding are extremely complex. Diltiazem has no effect on the binding of $^3$H-nimodipine until it reaches a concentration of approximately $10^{-7}$M, when it increases the specific binding of the $^3$H-ligand. The increase in binding reaches a maximum of 140% between $3 \times 10^{-6}$M and $10^{-5}$M diltiazem. The specific binding of $^3$H-nimodipine then decreases rapidly above $3 \times 10^{-5}$M diltiazem. Verapamil, on the other hand, displaces $^3$H-nimodipine at concentrations above $10^{-5}$M. However, the inhibitory effect of verapamil plateaus between $3 \times 10^{-5}$M and $10^{-5}$M. No further displacement is noted until concentrations above $3 \times 10^{-5}$M verapamil are obtained.

The apparent affinity constants ($K_i$) for a variety of calcium channel antagonists are given in Table 1. The 1,4-dihydropyridines, as well as sodium nitroprusside, display monophasic inhibition of $^3$H-nimodipine. This type of inhibition suggests that these drugs are competitive antagonists at the $^3$H-ligand binding site. Nimodipine and nitrendipine are essentially equipotent while nifedipine is approximately three times weaker in displacing $^3$H-nimodipine. Sodium nitroprusside displaces $^3$H-nimodipine but only at comparatively high concentrations. Propranolol, which has no known calcium antagonist properties,$^{19}$ is inactive in displacing $^3$H-nimodipine at concentrations to $10^{-5}$M.

Because of the atypical displacement curves of diltiazem and verapamil, apparent $K_i$ values cannot be determined. However, assuming the "plateau" in $^3$H-nimodipine binding represents a maximal pharmacological action, concentrations were determined which produce 50% of this effect ($ED_{50}$). As shown in Table 1, both verapamil ($250$ nM) and diltiazem ($260$ nM) are equipotent in terms of causing a shift in the binding parameters of $^3$H-nimodipine. As noted in Fig. 3, however, their pharmacological effects have opposite results on the specific binding of $^3$H-nimodipine: verapamil decreases the binding while diltiazem increases the specific binding of $^3$H-nimodipine.

**Table 1**

<table>
<thead>
<tr>
<th>Drug Effect</th>
<th>Apparent $K_i$ (nM)</th>
<th>ED$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>Nimodipine</td>
<td>0.29 ± 0.07</td>
<td>250</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>0.40 ± 0.2</td>
<td>250</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>1.0 ± 0.3</td>
<td>250</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>6000 ± 2000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Propranolol</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
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* $ED_{50}$ values were determined by using $0.1$ nM $^3$H-nimodipine in the presence or absence of three to 11 concentrations of each drug under standard assay conditions. $ED_{50}$ values were determined by log-probit analysis and apparent $K_i$ values were calculated from the equation $K_i = ED_{50}/(1 + 3^H$-nimodipine/$K_0$). The $K_0$ of $^3$H-nimodipine ($0.27$ nM) was obtained from Fig. 1. Values given are the means ± standard errors of four experiments, each performed in triplicate.

**Fig. 3.** Effects of calcium antagonist drugs on $^3$H-nimodipine binding to human frontal cortex membranes. $^3$H-nimodipine binding was obtained under standard assay conditions in the presence or absence of a variety of calcium antagonist drugs. Data given are the means of triplicate assays performed in a single experiment. Drugs studied were nimodipine, nitrendipine, nifedipine, diltiazem, and verapamil.

**Discussion**

The major finding of the present study is that the calcium antagonist, $^3$H-nimodipine, labels a high-affin-
ity saturable binding site in human frontal cerebral cortex. The binding site is also regulated by calcium with a maximal number of sites labeled in the presence of 1 to 100 mM calcium. Drug competition studies reveal monophasic inhibition by the 1,4-dihydropyridines but complex interactions with other calcium antagonists. These studies represent the first identification of $^3$H-calcium antagonist binding sites in human brain, and confirm and extend recent reports of specific calcium channel antagonist binding sites in non-human tissues. Thus, “calcium antagonist” drugs differ with respect to both potency and molecular site of action.

Binding studies in non-human tissues have been used to describe three classes of calcium channel antagonists. Class I agents are represented by the 1,4-dihydropyridines and display monophasic inhibition of $^3$H-ligand binding. The apparent $K_i$ of nimodipine and nifedipine also correlates with drug inhibition of certain physiological responses such as agonist-induced contractions of rabbit and canine basilar arteries. Class II agents include verapamil and methoxyverapamil (D600); these decrease the binding of $^3$H-calcium antagonists. Class III drugs, of which diltiazem is the prototype, increase $^3$H-ligand binding to calcium antagonist receptors. Thus, $^3$H-nimodipine binding in human brain also allows for the differentiation of three distinct classes of calcium antagonists.

Calcium channel antagonists have unique and selective effects on intracerebral vasculature and appear to be beneficial in the treatment of delayed ischemic neurological deficits from cerebral arterial spasm. During clipping of a ruptured aneurysm, topically applied nimodipine was found to cause vasodilatation. The observed dilatory effect was most marked in small vessels. In the recently completed controlled trial, oral nimodipine given orally was found to significantly prevent the occurrence of severe neurological deficits from spasm. Nimodipine did not, however, completely prevent the occurrence of neurological symptoms from spasm. Random cerebrospinal fluid levels of nimodipine were obtained from six patients in the study and averaged 1.8 nM. Assuming that the nimodipine binding site in the human cerebral arteries is identical to that of human frontal cortex with $K_i$ value of 0.27 nM, a concentration of 1.8 nM nimodipine should occupy 80% to 90% of the receptors under ideal conditions. In reality, the in vivo effective concentration of nimodipine for its physiological receptor site is probably somewhat higher due to tissue distribution, protein binding, and drug interaction with other competitors for the calcium channel recognition site. This point is illustrated by the $ED_{50}$ of 1.4 nM for nimodipine calculated by log-probit analysis of 50 nM serotonin-induced contractions of canine basilar artery segments in vivo. In the intact animal or human, the $ED_{50}$ is probably even greater.

In conclusion, high-affinity saturable $^3$H-nimodipine binding can be obtained in human brain membranes. The pharmacological characteristics of this recognition site suggest the direct labeling of the calcium channel antagonist receptor. The complex nature of the binding parameters confirms and extends studies in non-human tissues. Radioligand binding studies are a rapid and sensitive method that can be used in the pharmacological analysis of calcium channel antagonists. The results obtained by these in vitro studies should provide a useful guide for the selection of drugs and dosages in future clinical trials of calcium channel antagonists.

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